COMMUNICATION TO THE EDITOR

Effect of Airflow Rate on Yields of *Steinernema carpocapse* Az 20 in Liquid Culture in an External-Loop Airlift Bioreactor

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Abstract: Maximization of the contact between males and females is a key factor in the production of the nematode *Steinernema carpocapsae* in a bioreactor.

The influence of the airflow rate in male and female distribution and mass production in an external-loop bioreactor with a deceleration zone was studied. When operating at an airflow rate of 0.05 vvm, a high retention of females in the deceleration zone of the bioreactor was observed and a larger nematode productivity was obtained. At this aeration rate there was a higher proportion of males in that zone, which together with the lower circulation rate, increases the probability of encounters, thereby explaining the increase in productivity. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **72**: 369–373, 2001.
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INTRODUCTION

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*are soil-inhabiting lethal parasites of a broad range of insects which are used in biological control (Grewal and Georgis, 1998). They are used in the protected crops industry, where they are an effective alternative to chemicals for the control of a wide range of insects (Richardson, 1990) and they could be used on a large scale in integrated pest management, organic farming, and sustainable agriculture systems to control soil-borne insect pests (Ehlers, 1996).

The key feature of the *S. carpocapsae* life cycle is the symbiotic association with the bacterium *Xenorhabdus nematophilus*. The nematode infective juvenile (IJ) enters a susceptible host insect through natural body openings (mouth, spiracles, and anus) or directly through the insect cuticle (Peters and Ehlers, 1994) and invades its hemocoel releasing the bacteria. The bacteria multiplies, killing the insect by septicemia within 24–48 h of infection. The nematodes feed on the host tissue and cells of the symbiotic bacteria, develop into adults, mate, and reproduce within the host, often for multiple generations. When host nutrients are depleted, IJs are produced, which, upon exiting the cadaver, seek and infect new hosts.

Recent advances in our understanding of nematode nutritional requirements resulted in the development of in vitro mass production methods for *Steinernema*. However, these methods are time-consuming and cannot be easily implemented. Production costs are high, thereby limiting the use of nematodes to high-value crops. Thus, in order to reduce these costs, liquid culture systems have been actively investigated by a number of scientific and commercial organizations (Ehlers, 1996). Still, the effectiveness of these systems is not very high. In fact, attempts to produce nematodes using a stirred tank bioreactor or internal-loop airlift bioreactor resulted in yields that may range from 90×10^3 IJs/ml to 95×10^3 IJs/ml, respectively (Pace et al., 1986; Friedman et al., 1989), both authors realizing that nematodes can be severely affected by shear stress and by oxygen limitation.

Another problem can be the low mating rate. Previous work has shown that there are significant differences in physical properties, namely, size and density, between nematode males and females (Neves et al., 1996). These differences may hinder sexual contact in bioreactors, thereby reducing overall nematode productivity. Therefore, bioreactors with a nonconventional design must be considered, and among the several new types of bioreactors airlift systems are becoming more important (Siegel and Robinson, 1992).

In this work, we studied the ability to achieve nematode mass production by promoting sexual contact between males and females, using a specially designed external-loop airlift bioreactor under different airflow rates.

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MATERIALS AND METHODS

Bioreactor

The schematic diagram of the external-loop airlift used in this work is shown in Figure 1. It consisted of a Plexiglas bioreactor and was composed of a riser (R) with an inside diameter of 0.30 m and a downcomer with an inside diameter of 0.016 m. The ratio of the cross-sectional area of the downcomer (D) to the riser (AD/AR) was 0.28. The top of the riser—deceleration zone (DZ)—was of the cylindrical conical type, to facilitate the solid phase (nematodes) deceleration. The height and diameter of this enlarged part were 0.11 m and 0.06 m, respectively. All other dimensions are presented in Table I. The riser, downcomer, and connecting pipes were tubular and, in order to avoid a settling zone, the downmost connection pipe between riser and downcomer was inclined. This connection pipe configuration has been used previously by other investigators (Kawase, 1994). The airlift vessel contained an unaerated working volume of 0.5 l. Air was sparged into the bioreactor through a perforated plate in the base of the bioreactor and the airflow rate was measured with a calibrated rotameter.

Liquid Velocity

The liquid velocity (U_L) was determined using a pulse tracer technique as described by Chisti et al. (1988). As a tracer, a 1M NaOH solution was used, which was injected at the downcomer inlet. A pH electrode was placed near the exit of the downcomer and was coupled to a data acquisition system (PCL-812PG Enhanced Multi-Lab Card; Advantech Co.), which transmitted the signal to a personal computer. The liquid velocity inside the downcomer (U_{LD}) was calculated by dividing the average liquid path by the period elapsed between two adjacent pH maxima of the pulse propagation. Liquid velocities in the riser (U_{LR}) and in the deceleration zone (U_{LDZ}) were determined using the calculated value in downcomer (U_{LD}), and applying the continuity equation to the liquid phase (A_D, A_R, A_{DZ} represent the sectional area of the downcomer, riser, and deceleration zone, respectively):

$$U_{LR} = U_{LD} (A_D/A_R) \quad (1)$$

$$U_{LDZ} = U_{LD} (A_D/A_{DZ}) \quad (2)$$

Five replicates were made for each set of experimental conditions.

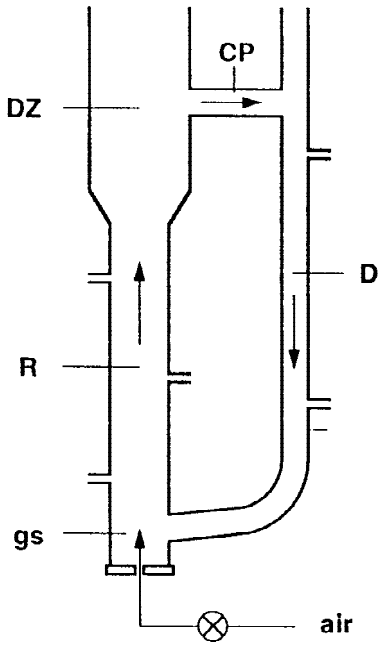

Figure 1. Schematic diagram of the experimental bioreactor; R (riser); D (downcomer); DZ (deceleration zone); gs (gas sparger); CP (connection pipe).

Table I. Characteristics of the external-loop airlift bioreactor.

Characteristics	Values
Scale	Laboratory
Total volume	0.60 l
Total length	0.60 m
Deceleration Zone	
Length	0.11 m
Inner diameter	0.06 m
Circulation Zone	
Riser	
Length	0.23 m
Inner diameter	0.032 m
Downcomer	
Length	0.55 m
Inner diameter	0.016 m
Connection Pipe	
Length	0.04 m
Inner diameter	0.016 m

Bioreactor Setup

The bioreactor was sterilized in situ with Betadine solution (10% v/v). The medium used was based in BEM medium (Jewell and Dunphy, 1995) and consisted of soyflour (20 g), yeast extract (10 g), egg yolk (10 g), corn oil (19.5 g), NaCl (5 g), KH2PO4 (2.5 g), distilled water (1,000 ml), pH 8.0. After preparation the medium was introduced into the bioreactor with a peristaltic pump.

Culture Conditions

Culture conditions were initially pH 8.0; working volume, 500 ml; airflow rate, 0.05 vvm, and 0.15 vvm; bacterial inoculum, 12.5 ml of 48 h of *X. nematophilus*; nematode inoculum, 25×10^4 IJs, corresponding to an initial concentration of 500 IJs/ml. The temperature was kept at 28°C during initial bacteria growth, reduced to 23°C during nematode culture. The bioreactor operated in batch mode for 15 days. Culture samples were collected from the bioreactor at selected times to check nematode viability and adult distribution. The final population of worms was determined by counting under a stereomicroscope. Five bioreactor batches were made for each of the tested conditions.

Adult Distribution in the Bioreactor

Steinernema carpocapsae Az 20 adult distribution in the bioreactor was determined when several airflow rates were applied to the system (0.01, 0.05, 0.09, 0.15 vvm). At each zone of the bioreactor and for each airflow rate an aliquot of 1 ml of the suspension was collected with a syringe. The number of males and females present in the aliquot was determined. For each zone and experimental situation, 10 samples of biomass were estimated.

Culture Conditions in Erlenmeyer Flasks

Flasks (250 ml) containing 30 ml of BEM medium were inoculated with 0.75 ml of a 48-h culture of *X. nematophilus*, placed on a 150 rpm orbital shaker at 28°C for 2 days, then inoculated with 500 IJs/ml. During nematode culture the incubation conditions were reduced to 23°C and 80 rpm. The final population was determined by counting under a stereomicroscope. Six replicates were made.

RESULTS AND DISCUSSION

The adult distribution among the different sections of the bioreactor at the four airflow rates tested is shown in Figures 2 and 3. For an airflow rate of 0.01 vvm the liquid circulation velocity (0.11 cms^{-1}) was lower than the sedimentation rate of males and females, 0.17 cms^{-1} and 0.37 cms^{-1}, respectively (Neves et al., 1996), and the adults settled at the bottom of the bioreactor, below the sparger. For the other tested airflow rates it was clear that male and female distribution was affected differently.

The distribution of males (Fig. 2) was independent of the airflow rate, whereas the female distribution pattern depended strongly on the aeration (Fig. 3). This is not surprising, considering the differences in physical properties, namely, size and density, between males and females. Indeed, *S. carpocapsae* females are much bigger than males (Poinar, 1979) and present a different density (Neves et al., 1996). These differences lead to different sedimentation rates and, under certain airflow conditions, to different

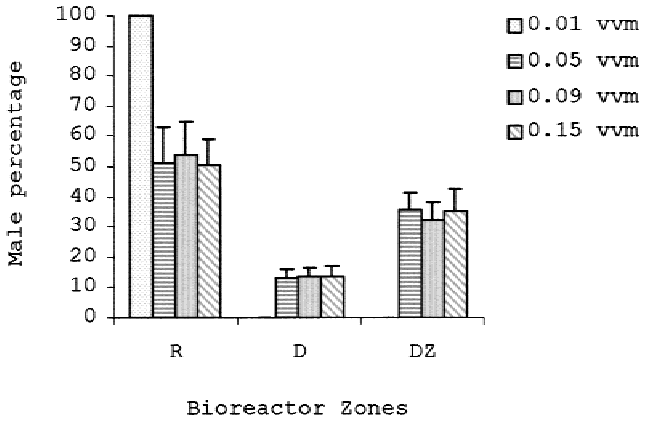

Figure 2. Male distribution in the bioreactor at different airflow rates.

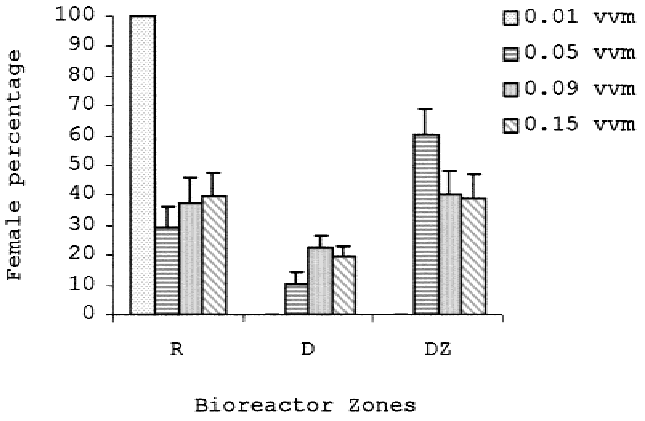

Figure 3. Female distribution in the bioreactor at different airflow rates.

distribution patterns. At 0.05 vvm, the liquid circulation velocity (0.30 cms^{-1}) was slightly lower than the sedimentation rate of the nematode females in Tyrodes' solution (0.37 cms^{-1}) (Neves et al., 1996). Under these conditions, it was possible to retain 60.6% of all the females in the deceleration zone. When the airflow rate was 0.09 vvm, females tended to distribute evenly between the different sections of the bioreactor and this tendency became more evident at 0.15 vvm. At this airflow rate, the liquid circulation velocity increased to 0.62 cms^{-1} and the percentage of females retained in the deceleration zone decreased to 38.9%. Therefore, it may be concluded that higher airflow rates tend to bring about uniform distribution.

It is important to note that these results show the heterogeneity of adult distribution when certain airflow rates are used. These experimental observations appear to agree with similar findings by Siegel et al. (1989) and Assa and Bar (1991). This characteristic can be very important when the solid phase of the bioreactor is composed of particles with different physical properties, such as nematodes in which males and females have different sizes and densities (Neves et al., 1996). In order to maximize egg productivity, the highest possible number of females must be fertilized, so it is important to concentrate the females in a region of the bioreactor in order to favor mating between the retained females and the circulating males.

After 15 days of operation, at 0.05 vvm the IJs production in the bioreactor reached 30×10^6 (60×10^3/ml). On the other hand, when an airflow rate of 0.15 vvm was used the maximum number of IJs obtained was just 19×10^6 (38×10^3/ml)—a 39% reduction, which proved to be significantly different at the 5% confidence level (Table II).

The first conclusion to be drawn is that the culture in Erlenmeyer flasks probably suffers from oxygen limitation, since it is the one where the yield and the aeration were the lowest (Table II). In turn, the results obtained with an aeration rate corresponding to 0.05 vvm are relevant, since inoculation conditions were the same for all the experimental runs. The initial population used for every run was collected from the same batch of dauer larvae. Therefore, as the cul-

Table II. *Steinernema carpocapsae* mass production under different culture conditions.

	Airflow rate (vvm)	Inoculum (IJ/ml)	Final (IJ/ml)		Rf
Bioreactor	0.05	500	60000	a	120
	0.15	500	38000	b	76
Erlenmeyer	—	500	14700	c	29

Rf (reproduction factor) = Final concentration/inoculum concentration.

ture medium was the same in all circumstances, all the individuals had the same chance to multiply.

One could think that the female proportion could vary from one inoculum to another.

However, the total population used in each case was 25×10^4 individuals in 500 ml of medium (500 IJ/ml). For large samples, such as those used in this work, the average proportion of females in each sample follows a normal distribution (Ludwig and Reynolds, 1988). If we consider m the average proportion of females in a sample and μ the true proportion of females in the population, then the average proportion of females in any sample large enough will be expected to fall inside the interval:

$$m - \mu = \pm Z\sigma/n^{0.5} \quad (3)$$

The standard normal distribution variable Z is less than 4 (Ludwig and Reynolds, 1988).

Even for $Z = 4$, the probability that m lies in the above interval is 99.994%. Considering that the sample size is $n = 25 \times 10^4$ and replacing the numerical values in Eq. (3), we finally get the expression:

$$m - \mu = \pm 4\sigma/500 = \pm 0.008\ \sigma \quad (4)$$

There will thus be a very high probability (99.994%) that the female proportion in the samples will stay very close to the true female proportion. Therefore, the large increase observed in the yield cannot possibly be attributed to sex ratio variations between samples. What could explain this increase in the final yield could be 1) an increase in the mating rate; 2) an increase in the number of eggs per fertilized female; 3) an increase in the mating rate per male; or 4) a reduction in the female gestation cycle. All the explanations but the increase in mating rate would imply drastic deviations from what is currently accepted for the biology of this nematode, especially since no changes were introduced in the culture medium composition.

On the other hand, as can be seen in Figures 2 and 3, with the exception of 0.01 vvm, the region where the maximum number of females is observed—always above 40% of the total—in all aeration rates is the deceleration zone. Furthermore, the aeration for which the number of males is maximized in DZ corresponds to 0.05 vvm. On the contrary, for 0.15 vvm a smaller number of males will be present in DZ. It is thus quite reasonable to suppose that the encounters between males and females are favored for an aeration rate of 0.05 vvm. Since it was verified that the yield was maximum at 0.05 vvm, it is also probable that mating occurs mainly in DZ.

Finally, it is known that the reproduction of this nematode is strictly sexual, with no reported cases of hermaphroditism. Since in a defined medium the number of eggs laid per female is quite constant, the only possible explanation for an increasing number of individuals is the increase in the number of fertilized females.

The results show that the aeration rate could not be directly responsible for the production differences, since a lower aeration rate induced higher production, but can be associated with an increase in the mating rate due to a lower liquid circulation velocity. Actually, when using an airflow able to generate a liquid circulation velocity similar to the female sedimentation rate, the enlarged part of the riser—deceleration zone—can function as a "gyneceum" where the mating rate is enhanced. In fact, the results show that when the females are more concentrated in the deceleration zone (0.05 vvm) the yield, i.e., the reproductive factor (Rf = final concentration / inoculum concentration) is higher (Rf = 120). On the contrary, when the higher airflow (0.15 vvm) gave rise to a higher female circulation, the Rf decreased to 76.

The assessment on pathogenicity was done by determining the LD_{50}, i.e., the number of IJs required to kill 50% of the insects. For IJs produced in the bioreactor, 20.7 IJs were needed to kill 50% of the insects, whereas for those produced in vivo, only 16.2 IJs were needed. This means that a rather high level of lethality was kept in the present experimental conditions.

CONCLUSIONS

Although a uniform distribution of the solid and liquid phases throughout the airlift vessel is generally sought, it is sometimes desirable to create, in particular cases, nonhomogenous distribution patterns of the solid phase. By associating the difference in the physical properties of the components of the solid phase—male and female nematodes—with an adequate design of the bioreactor, it is possible to develop a more efficient system for nematode production in submerged cultures. The sexual contact between adults was improved by creating a zone of low liquid velocity where a high concentration of females was maintained, thus improving the mating opportunities with males that circulate through the bioreactor.

The results obtained show that the airlift system proposed in this work is more efficient than those reported in other works. The advantage of the proposed reactor design probably becomes clearer if the yield achieved is compared with those obtained with other *S. carpocapsae* production systems: Rf = 95 in Friedman et al. (1989), internal-loop airlift bioreactor; Rf = 45 in Pace et al. (1986), stirred tank bioreactor; and Rf = 29 in 250 ml Erlenmeyer flasks. Furthermore, the slight reduction found in the IJs pathogenicity implies that future efforts should be focused on the improvement of the artificial culture medium.

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